Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Available online at www.inia.es/sjar
http://dx.doi.org/10.5424/sjar/2013113-3987

Spanish Journal of Agricultural Research 2013 11(3): 842-854
ISSN: 1695-971-X
eISSN: 2171-9292

Transcriptome analysis in *Ceratitis capitata* to unveil genes involved in ageing-maturation process

V. San Andrés[1], P. Castañera[2] and B. Sabater-Muñoz[1]*

[1] *Unidad Asociada de Entomología del IVIA-CIB (CSIC). Centro de Protección Vegetal y Biotecnología. IVIA. Ctra. Moncada-Náquera, km 4,5. 46113 Moncada (Valencia), Spain*

[2] *Unidad Asociada de Entomología del IVIA-CIB (CSIC). Departamento de Biología de Plantas. CIB-CSIC. C/ Ramiro de Maeztu, 9. 28040 Madrid, Spain*

Abstract

The sterile insect technique (SIT) is widely used in integrated programmes against the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). Information on the age distribution of insects, and more particularly, the knowledge of wild female reproductive status (mature or not) at the time of the sterile male release is one of the key factors for the success of the SIT. In recent years, sequencing analysis has become an important tool in molecular biology. In this work we present a genome-wide expression analysis based on SSH (substractive sequence hybridization) and EST (expressed sequence tag) sequencing and macroarray expression analysis to identify signature genes related to the ageing-maturing process in *C. capitata*, leading to the successful identification of new putative candidate genes of reproductive status in medfly that would serve as molecular markers for ageing. We have sorted out 94 unigenes from 873 single-pass ESTs, of which 57% have homology with known genes. Ageing-maturing process in *C. capitata* presents a marked expression pattern accompanied by the increase of transcription level of genes involved in reproduction (vitellogenins, chorion proteins and male-specific serum proteins). Other identified cDNAs (43%) with a differential expression pattern would be also candidates but deserve further studies, as they belong to the unknown function class.

Additional key words: ageing; expression profile; genome analysis; medfly; macroarray; sexual maturation; SSH.

Introduction

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is a highly polyphagous insect pest (Liquido *et al.,* 1991; White & Elson-Harris, 1992). Its invasive behaviour has led to its consideration as a major quarantine pest in many countries (Malacrida *et al.,* 2007) and has led to the development of extensive control programmes. In Spain, current medfly control management is mainly based on the use of insecticides; nevertheless the demand for insecticide-free fresh fruit is encouraging the use of environment-friendly methods. The sterile insect technique (SIT) has proven to be an effective and environmentally friendly control method against medfly (which has become a model organism in this technique) and has exponentially grown world-wide in the last years (Hendrichs *et al.,* 2005). As such, an area-wide suppression programme was initiated in 2006 in Spanish Mediterranean citrus areas (San Andrés *et al.,* 2007). The success of a SIT programme depends greatly on the lifespan of mature males released and on the successful mating with wild females. The knowledge of wild female reproductive status (immature, gravid or old) at the time of the sterile male release and the establishment of optimal sexual maturity in sterile released males are key factors for the success of the SIT. The functional maturation of gonads and the onset of mature sexual behaviour are a result of an orderly and timely transition of genome expression through

* Corresponding author: bsabater@ivia.es; b.sabater.munyoz@gmail.com
Received: 16-01-13. Accepted: 16-07-13.

Abbreviations used; EST (expressed sequence tag); GO (gene ontology); RACE RT-PCR (rapid amplification of cDNA ends by reverse transcription-polymerase chain reaction); SCSIE (Servicio Central de Soporte a la Investigación Experimental); SIT (sterile insect technique); SSH (substractive sequence hybridization); Vg (vitelogenin).

developmental stages. This genome expression orchestration is mainly unknown in the medfly but some biochemical pathways are known in diverse insect species, on which *corpus allatum* and juvenile hormones play a central role (Moshitzky *et al.,* 2003 and references herein).

Ageing is a process in which the progressive loss of function in multiple tissues (including a reduction in reproduction capacity) results in the increasing probability of death (Kirkwood & Austad, 2000), thus ageing and reproductive status are linked processes. However, ageing is one of the most complex biological processes being determined by both, genetic and environmental factors. In *C. capitata* sex-specific life span patterns have been described as resulting from an underlying "constitutional" longevity minus the deleterious effects of reproductive biology (*i.e.* progeny production and effects of reproductive hormones), and sex-related behaviour (*i.e.* seeking and competing for mates, or territorial defence) (Novoseltsev *et al*., 2005; Carey *et al.,* 2006, 2008). Despite the central importance of insect population age structure, relatively few methods have been developed to measure insect age in the field. Both physiological or chronological age could be estimated by the identification of ovarian development in females (Tyndale-Biscoe, 1984), the progressive layering of hydrocarbons in insect cuticles (Desena *et al.,* 1999; Gerade *et al.,* 2004), the use of pteridines (Camin *et al.,* 1991; Mullens & Lehane, 1995; Penilla *et al.,* 2002) or the use of behavioural trait biomarkers (*i.e.* supine behaviour) (Papadopoulos *et al.,* 2002). More recently, the transcriptional profiling method of ageing, based on genes that display age-dependent expression, have been applied in mosquito (Cook *et al.,* 2006, 2007).

Single gene mutations that prolong the longevity are most useful to gain insights into the molecular mechanisms of ageing process and longevity determination with its link with reproductive status. Genetic analyses of yeast, nematode, fly, and mouse have uncovered a number of genes (*i.e.* superoxide dismutase, methuselah, *daf, hsp70*) whether mutated or misexpressed, increase the lifespan of these short-lived model organisms and support the general idea of free-radical hypothesis of ageing (Kim, 2008).

Genome-wide expression analysis has emerged as a powerful tool that can be used for the identification of signature genes that behave similarly under particular conditions and time points or under particular conditions, and which can be used in very different ways to analyze large-scale gene expression across species involved in ageing (McCarroll *et al.,* 2004). In a fashion similar to microarrays, the macroarrays have been used to perform this genome-wide expression analysis (Alberola *et al*., 2004). This analysis requires the knowledge of the target genome or the use of model organisms. If the target organism is not a model one (its genome is probably unknown), or the number of identified genes is low (as in ageing), the expression analysis would become hard to perform. One way to solve this problem is the use of suppressive substractive hybridization technique (SSH) (Diatchenko *et al.,* 1996). The basis of this technique is the comparison of two expression conditions with the objective of identifying differentially expressed genes. Furthermore, SSH can identify rare transcripts (low-abundance differentially expressed cDNAs) and is a highly effective method for generating subtracted cDNA libraries.

We report here on a genome-wide expression analysis —from newly emerged, mature and old non virgin medfly adults— to unveil the medfly genes involved in ageing-maturity by SSH and macroarray hybridization. We used target genes previously described as being related to ageing as well as the new candidate genes obtained by SSH. The final aim is the identification of new putative candidate genes for longevity and/or for determination of reproductive status in medfly.

Material and methods

Unless otherwise indicated, all molecular techniques and solutions were performed as described by Sambrook *et al.* (1989).

Medfly time point samples

Adults of *C. capitata* were collected from a laboratory colony maintained at the IVIA (Valencia, Spain) since 2002. In order to obtain a cohort of adults, approximately 2,000 pupae were separated in a Perspex cage (20 cm × 20 cm × 20 cm) and adults were allowed to emerge. To obtain a cohort of adults (< 24h), newly emerged adults were separated and all unhatched pupae were removed and discarded. After emergence, flies had *ad libitum* access to a diet consisting of a mixture of sugar and hydrolyzed yeast (Biokar Diagnostics Co., Pantin, France) (4:1; w:w) and water.

According to the average lifespan of this strain (17 days, unpublished data) and their biological status, the individuals are grouped in three categories: 1) immature adults (< 12 h-old), 2) mature (5 days-old adults), and 3) old (15 days-old adults). Once newly emerged adults were separated, both males and females were kept together in a single ventilated perspex cage (20 cm × 20 cm × 20 cm). For each of the three groups, 20 females and 20 males were fast-frozen and maintained at –80°C until used. Three biological replicates of each biological status course were performed separately in time for the macroarray assay.

RNA extraction

Total RNA was isolated using the TRI-Reagent® protocol (Sigma-Aldrich Co., St. Louis, MO, USA) from 10 adults, 5 males and 5 females of *C. capitata* from each age group (0, 5 and 15 days-old). The RNA concentration was measured with the Nanodrop ND-1000 (Agilent Tech. Inc., Palo Alto, CA, USA), and the quality verified in a 1% D1 low (electroendosmosis) EEO agarose gel (Pronadisa, Sumilab S.L., Madrid, Spain).

Suppressive substractive hybridization (SSH)

SSH procedure (Diatchenko *et al.,* 1996) was applied by using PCR-Select™ cDNA substraction kit (Clontech, Palo Alto, CA, USA). Two micrograms of RNA from each age group were used as source for cDNA and used as tester or driver in a circular substraction scheme (t0/t5, t5/t15, t15/t0; tester/driver) for each gender (Fig. 1). Substracted cDNAs were cloned into pGEM-T easy (Promega Biotech Ibérica S.L., Madrid, Spain) and transformed into DH5α electrocompetent *E. coli* cells (Invitrogen S.A., Barcelona, Spain) to obtain the subtracted cDNA libraries.

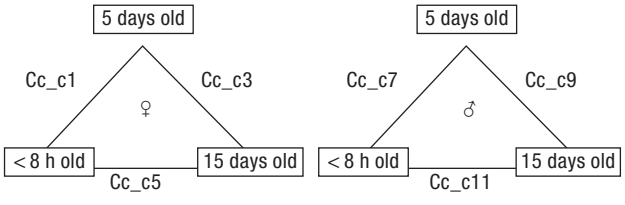

Figure 1. Medfly SSH libraries construction. Each library is constructed using one age condition (indicated in hours or days for each gender) as tester and another age point as driver. The driver source is indicated by the arrow head. Names of libraries are close to their corresponding arrow.

Library screening

Each library was plated on selective LB agar plates and white colonies were transferred to v-well plates containing 150 μL of liquid TB-glycerol medium with 50 μg mL^{-1} ampiciline. Cultures were set at 37°C, overnight without agitation. Plates were subjected to colony PCR (Sabater-Muñoz *et al.,* 2006). PCR products were purified with Sephadex G-50 superfine (GE-Amersham Healthcare, Chalfont St. Giles, UK) and verified by gel electrophoresis on a 2% agarose gel (Pronadisa). Cultured plates were stored at –80°C till used.

Size selected PCR products were directly sequenced at the Servicio Central de Soporte a la Investigación Experimental (SCSIE) from University of Valencia, using Bigdye® v3.1 chemistry (Applied Biosystems, Foster City, CA, USA) with primer T7 in 1/16 of the recommended reaction volume (single pass sequence reading).

Unigene set establishment

Electropherograms were checked and assembled into contigs by using Staden package software 1.6.0 version (Staden *et al.,* 2000) for each subtracted library. Each consensus sequence was compared to GenBank by blast using tblastx as implemented in NCBI web page to assign gene function (Altschul *et al.,* 1997). Sequences were also compared to those EST (expressed sequence tags) available of *C. capitata* in dbEST (GenBank: FG068301–FG089553; Gomulski *et al.,* 2012). Gene ontology (GO) was established by assignment of each identified gene function into GO browser. The sequences reported in this work have been deposited into GenBank (dbEST) at NCBI under accession number TSA117765.

All identified gene, gene product or putative ORF (open reading frame) were compared to all other by aligning sequences two by two with bl2seq algorithm at NCBI web page. Consensus sequences from same or different substracted libraries that coded for the same gene were aligned to the *D. melanogaster* ortholog or to the corresponding gene from medfly.

After these comparisons, a medfly unigene set was established and cultured again in v-well plates as des-

Table 1. Primers and probe size for ageing-related genes and controls used, specific for *Ceratitis capitata*

Gene name	Origin sequence	Forward primer	Reverse primer	Tm (°C)	Amplicon size (bp)
RpS21	AJ507827	GGA GAA CGA CGC CGG TGA ATC TG	GCC AAA CGC ACA ATA CAA TCA TC	55	218
RpO[a]	Y18444	TCC AGG CTC TCT CCA TAC CA	CGA CTT TGT CAC CAC CAG GCT TC	60	90
SOD1	M76975	TGC AAA GTC TCC CGT CCT AGT CAC	CAA ATT CCA ATG ACG CCA CAA CC	55	382
SOD2	L35494	ACG TGG AGG GTC CCG TAG TGA AG	CTG CGC CTG CAT TGC CAG TCT TC	52	404
Catalase	This work[b]	GAA AAG GTG CAT AAA CGT AC	GCT GGC GTT GCT TAA ACT CG	50	1,100
*hsp*70	Y08955	ATT GCT GGT TTG AAT GTG TTA CG	TTG TCG CCA CTG AGA ATA GCT GC	57	664

[a] Primers and amplification conditions as in Magaña *et al.* (2008). [b] Obtained by RACE RT-PCR with degenerate primers, cDNA cloned into pGEM-T Easy and sequenced. Primers were designed on the obtained sequence.

cribed. This new gene collection was used as source for probes for the expression assay.

Macroarray printing, hybridization and data analysis

Additional probes were generated by RT-PCR with specific primers from some of the characterized genes from medfly which are involved in ageing process in model organisms. These genes are: SOD1, SOD2, *hsp*70 and catalase. Two medfly genes, RpO and RpS21, were used as references – see Table 1 for specific primers sequence and amplicon sizes. Catalase probe was obtained from a cDNA clone generated during this work by using RACE RT-PCR kit (Invitrogen) following manufacturer instructions with a degenerated primer (catDIR-d1: 5' ATT CCR GAR CGW GTN GTR CAY GC-3') designed from an alignment of catalase genes from *Drosophila melanogaster* (X52286), *Apis melifera* (AF436842), *Bombix mori* (AB164195) and *Anopheles gambiae* (AY505416). The 1.2 kb PCR fragment was cloned into pGEM-T Easy (Promega) following manufacturer instructions. After clone sequencing, primers (Table 1) were designed to amplify the medfly catalase gene from the pGEM-T cloned copy to be used as probe in the macroarray. SSH probes were generated by PCR using the unigene collection created which contained 94 clones (see Table 2 and 3).

BioGrid (BioRobotics Li., Cambridge, UK) was used as the spotting robot. Macroarrays were made by printing the PCR products (without purification) onto a positively charged nylon membrane (Amersham Hybond N+). Printing was done with a 384-pinhead printer, consisting of regular 4 × 4 spots per pin, yielding a total of 360 available positions distributed in 12

Table 2. Summary of the *C. capitata* EST libraries. The total number of high quality sequences after trimming of vector and short or poor quality sequences, ribosomal or not genomic medfly origin, the sequence length average, the number of singletons ("unique" clones), contigs (more than one clone with contiguous nucleotide sequences), and unigenes (the number of singletons and contigs) per library, as well as the number of ESTs that had BLASTX matches to the NCBI non-redundant database

Library	Total no. of clones	Total no. of selected clones[a]	ESTs of high quality sequences	Sequence average (bp)[b]	No. of contigs	No. of singletons	No. of unigenes[c]	No. of unigenes matching GenBank database sequences	No. of unigenes without match
Cc_C1	496	100	93	448	10	7	17	12	5
Cc_C3	592	193	154	321	10	15	25	17	8
Cc_C5	592	237	114	411	5	5	10	4	6
Cc_C7	592	116	56	339	5	6	11	4	8
Cc_C9	592	137	37	273	4	13	17	12	5
Cc_C11	592	90	64	329	7	7	14	10	4
Total clones	3456	873	518						

[a] > 500 bp, [b] after trimming. [c] unigenes represent putative unique sequences.

Table 3. Unigen set printed in macroarray membranes

Unigen ID	BlastX	bp	*p*-value	GO[a]	GenBank[b]	NSC[c]
CC1P1B6	Low homology	263	—	—	—	1
CC1P1H4	Unknown function	284	$7 \cdot 10^{-42}$	Unknown function	FG086494	2
CC1P1H5	Unknown function	263	$3 \cdot 10^{-26}$	Unknown function	FG079286	1
CC1P4A10	Cuticle protein	564	$3 \cdot 80 \cdot 10^{-5}$	Cell development	AE003618	9
CC1P4A4	Vitelogenin precursor	567	$3 \cdot 20 \cdot 10^{-57}$	Cell development	X54662	1
CC1P4B2	Cuticle protein precursor DACP	361	$3 \cdot 40 \cdot 10^{-5}$	Cell development	AE003618	7
CC1P4C5	Low homology	205	—	—	—	2
CC1P4C6	Low homology	402	—	—	—	2
CC1P5A2	Hexamerin	879	$1.6 \cdot 10^{-46}$	Regulation of biological characteristic	U89789	17
CC1P5F5	Flotillin	309	$5.3 \cdot 10^{-39}$		AE003810	2
CC1P5G3	Hexamerin	599	$3.7 \cdot 10^{-47}$	Cellular adhesion	U89789	7
CC1P5H1	Low homology	294	—	Regulation of biological characteristic		9
CC1P6A9	Actin 1	447	$9.20 \cdot 10^{-44}$	Cell motion	X05185	3
CC1P6G4	Unkown funtion	575	$9 \cdot 10^{-53}$	Unknown function	FG082788	1
CC1P7C2	Similar to cuticle protein	393	$6 \cdot 10^{-62}$	—	GD22471	1
CC1P7G7	Low homology	322	—	—	GD22471	3
CC1P7H4	Low homology	215	—	—	—	1
CC1P8F7	Myosin light chain 2	834	$6.40 \cdot 10^{-91}$	Cell motion	M28643	3
CC1P8F8	Low homology	402	—	—	—	1
CC3P1A7	Putative enolase	431	$6.00 \cdot 10^{-29}$	Cellular metabolic process	AY725787	1
CC3P1B8	Cos38 gene	154	$1 \cdot 10^{-58}$	Cell development	X55886	1
CC3P1C10	Vitellogenin-2 precursor	519	$7.00 \cdot 10^{-62}$	Cell development	X54662	20
CC3P1C12	EF2	519	$4.00 \cdot 10^{-94}$	Cellular metabolic process	AY064104	38
CC3P1H11	Low homology	208	—	—	—	2
CC3P1H8	Guanine nucleotide-binding protein G(i) subunit alpha 65A	500	$5.00 \cdot 10^{-19}$	Cell communication	M23094.1	1
CC3P9B6	Similar to *D. melanogaster* CG12775 (RpL21)	453	$9.00 \cdot 10^{-56}$	Biosynthetic process	AY232061	1
CC3P9D4	Chorion protein s38	472	$2.00 \cdot 10^{-15}$	Cell development	X55886	11
CC3P9E8	Unknown function	231	$4.00 \cdot 10^{-6}$	Unknown function	XM_002009735/GI15052	11
CC3P10B12	Vitellogenin 1(gamma)	727	$2.00 \cdot 10^{-161}$	Cell development	X54661	17
CC3P11D3	Low homology	297	—	—	—	1
CC3P10H2	Low homology	196	—	—	—	1
CC3P11A11	Low homology	112	—	—	—	1
CC3P11E1	Trypsin-like serine protease precursor	297	$3.00 \cdot 10^{-12}$	Cellular metabolic process	AAF91346	1
CC3P12E3	Low homology	307	—	—	—	2
CC3P11H2	Chorion protein s19	598	$5.00 \cdot 10^{-10}$	Cell development	AJ251919	16
CC3P12H1	GA15316-PA (EF2)	231	$2.00 \cdot 10^{-30}$	Cellular metabolic process	CH379062	1
CC3P13H2	Low homology	137	—	—	—	2
CC3P14H12	Low homology	198	—	—	—	1
CC3P14H5	Chorion protein s36	143	$2.00 \cdot 10^{-8}$	Cell development	X51342	1
CC5P15A7	Low homology	203	—	—	—	1
CC5P15D4	Low homology	755	—	—	—	87
CC5P15G7	s19 chorion protein	125	$4.3 \cdot 10^{-12}$	Cell development	Y08914	1
CC5P16F7	Vm26Ab gene product	206	$6.1 \cdot 10^{-15}$	Cell development	AE003612	1
CC5P16H12	CG6391 gene product	449	$1.1 \cdot 10^{-6}$	Cellular metabolic process	AE003546	3

Table 3 (cont.). Unigen set printed in macroarray membranes

Unigen ID	BlastX	bp	*p*-value	GO[a]	GenBank[b]	NSC[c]
CC5P17H6	Low homology	400	—	—	—	2
CC5P19B2	Low homology	220	—	—	—	1
CC5P19D5	CG5284 gene product (chloride channel)	735	$4.3 \cdot 10^{-101}$	Localization	AE003528	1
CC5P19H4	Low homology	653	—	—	—	15
CC7P2E8	Low homology	214	—	—	—	2
CC7P2F9	Low homology	766	—	—	—	1
CC7P21A12	Low homology	106	—	—	—	1
CC7P21C10	CG6803 gene product	333	$3.10 \cdot 10^{-50}$	Cell development	AE003707	8
CC7P21D1	Actin A3	449	$2.70 \cdot 10^{-74}$	Cell motion	X97614	12
CC7P21F11	Low homology	272	—	—	—	1
CC7P21G10	Low homology	144	—	—	—	1
CC7P23D6	Low homology	369	—	—	—	2
CC7P23C9	CG8736 gene product	292	$4.90 \cdot 10^{-6}$	Unknow function	—	1
CC7P23A4	CG7203 g ene product	230	$1.00 \cdot 10^{-5}$	Unknow function	—	1
CC7P26H7	ATP synthase F0 subunit 6	609	$2.20 \cdot 10^{-88}$	Cellular metabolic process	AJ242872	2
CC9P3F4	Low homology	138	—	—	—	1
CC9P27H4	Low homology	131	—	—	—	1
CC9P29D11	Myosin heavy chain 3, muscle	462	$1.70 \cdot 10^{-69}$	Cell development	X53155	1
CC9P30B3	Low homology	106	—	—	—	3
CC9P30C8	Low homology	239	—	—	—	1
CC9P30D3	Male specific serum polypeptide β1	118	$1.7 \cdot 10^{-17}$	Response to chemical stimulus	Y19146	1
CC9P31D3	Low homology	331	—	—	—	1
CC9P32H1	ATP synthase F0 subunit 6	610	$1.50 \cdot 10^{-89}$	Cellular metabolic process	AJ242872	10
CC11P3C8	Male specific serum polypeptide β2	377	$4.6 \cdot 10^{-56}$	Response to chemical stimulus	Y19147	6
CC11P33B7	Low homology	114	—	—	—	1
CC11P33D7	Transferrin precursor	629	$4.80 \cdot 10^{-31}$	Response to chemical stimulus	AF063449	2
CC11P33H8	Low homology	122	—	—	—	1
CC11P35B12	Male specific serum polypeptide β2	194	$2.30 \cdot 10^{-22}$	Response to chemical stimulus	Y19147	1
CC11P35B6	Low homology	343	—	—	—	27
CC11P35F10	Male specific protein	274	$2.30 \cdot 10^{-15}$	Response to chemical stimulus	Y08954	2
CC11P35F8	Male specific serum polypeptide β1	385	$1.4 \cdot 10^{-40}$	Response to chemical stimulus	Y19144	1
CC11P36F11	Low homology	220	—	—	—	1
CC11P37A3	cytochrome b	605	$1.5 \cdot 10^{-86}$	Cellular metabolic process	AJ242872	1

[a] GO: gene ontology. [b] GenBank accesion number of the most similar sequence to the described EST. [c] NSC: number of sequences per contig.

blocks of 3 columns and 10 rows. Solid pins of 0.4-mm diameter were used. These pins spot 20 nL each time. PCR products were spotted five times so that each spot contained 20-30 ng DNA in a diameter of approximately 0.6 mm. Gene probes were printed in triplicate. The chips contained as positive control spots and spikes 5 triplicates of pGIBS-THR, pGIBS-PHE and pGIBS-LYS (cloned genes from *Bacillus subtilis*, from American type culture collection ATCC 87484, 87483 and 87482 respectively). Negative control spots included four replicates of 0.2 ng *Escherichia coli* genomic DNA. Therefore, 294 positions were used for

gene probes, 57 for control probes and 9 remained empty. Membranes were kept humid by setting them onto three Hybond blotting- paper sheets soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH). After printing, the membrane was neutralized with 1.5 M NaCl, 0.5 M Tris/HCl (pH 7.2), 1 mM EDTA (pH 8.0) for 1 min. Membranes were kept on filter paper until complete dryness.

Total RNA extraction from each time point for each biological replicate of medfly females and males were performed as described previously. Three medflies were used for each time point. Radioactive sample labelling including spikes, macroarray printing and hybridization, scanning and analysis were performed at SCSIE (Alberola *et al.,* 2004). A total of 6 membranes were printed and 18 hybridizations performed. Membranes were sweep rotated for each time point, and all samples from the same time point were hybridized at the same time.

Reproducibility of the replicates was tested using ARRAYSTAT software (Imaging Research Inc.) considering the data as independent and allowing the program to take a minimum number of valid replicates of two in order to calculate mean values for each cDNA. Application of a Z-test for independent data provided the differences in individual cDNA expression between two time points. A *p*-value of 0.05 and the false discovery rate method were used to monitor the overall false-positive error rate. Only those significantly expressed cDNAs were considered to perform a cluster analysis in order to arrange cDNAs according to similarity in pattern of gene expression. Cluster analysis was performed with the Self Organising Tree Algorithm SOTArray tool included in the Gene Expression Pattern Analysis Suite v 3.1 (GEPAS) (Herrero *et al.,* 2003) available at the CIPF Bioinformatics Unit (http://gepas3.bioinfo.cipf.es) using the linear correlation coefficient among the six-variables vectors as distance between genes.

Results

Substractive EST collection

The SSH performed resulted in a total of 3,456 independent cDNA clones isolated from the six cDNA libraries from which 873 clones were single-pass sequenced. Table 2 shows the characteristics of each library.

The known or putative functional assignments were based on the similarity of the deduced protein sequences to previously-described molecules deposited in the nr or EST database at NCBI.

The 518 ESTs obtained from all libraries were assembled into 94 unigenes after eliminating sequences corresponding to *D. melanogaster* C virus or to ribosomal RNAs. The number of ESTs per contig ranged from 1 to 87. The majority of the contigs were formed by less than 5 ESTs, being the singleton the most abundant class.

Globally, the largest set of genes was assigned to the unknown function or low homology class. Forty EST unigenes (43%) did not show similarity with known proteins (named as low homology class or unknown function); the 57% of the unigenes exhibited significant protein homology to previously identified or putative proteins in the database. The ESTs with significant protein homology were sorted by their biological process into 11 groups as the result of GO annotation: cell development (19%), response to chemical stimulus (17%), cellular metabolic process (7%), unknown function (5%), cell motion (4%), regulation of biological characteristic (2%), cellular adhesion (2%), protein transport (2%), cell communication (1%), biosynthetic process (1%), and localization (1%). These ESTs were classified differently among libraries according to their GO.

Transcriptional profiling

The new consensus sequences obtained by the comparison of the entire consensus ESTs by aligning sequences two by two with bl2seq algorithm at NCBI web page resulted in a new medfly unigen set collection which was used as probes in the macroarray (Table 3). All ESTs were submitted to the EST division of GenBank with accession number TSA grp117765 and can be accessed at the NCBI EST database.

The Z-score normalization (which preserves the original relative dispersion) allowed the comparison of all these genes for common profiles. Age-regulated ESTs were defined as those showing significant differences with the Z-score test using the age point zero (< 12 hr, newly emerged) as reference for the comparisons between the age points. This test indicated that 87.6 and 78.4% were significantly differential expressed in females and males respectively. The rela-

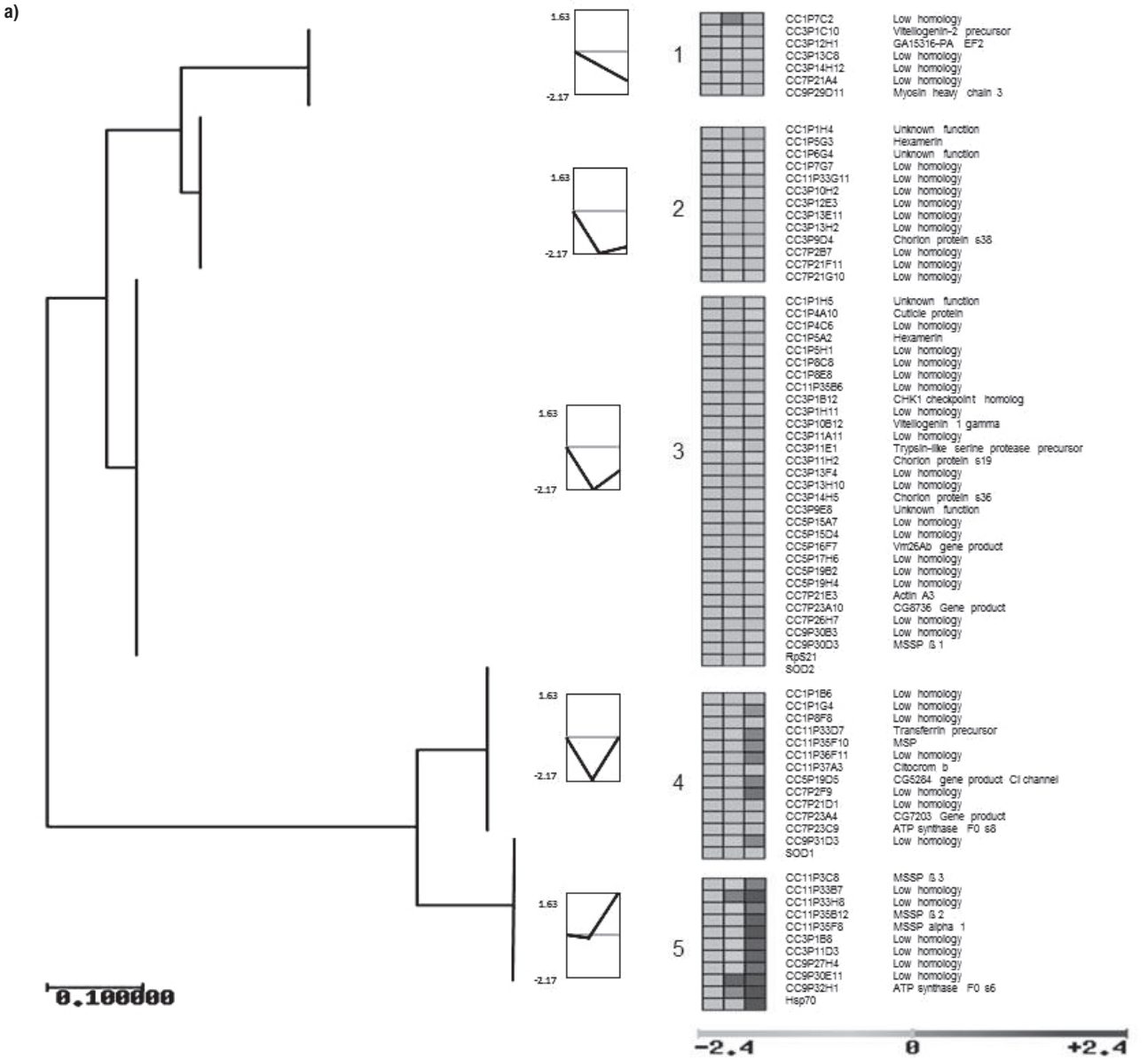

Figure 2. Hierarchical clustering of differentially expressed *C. capitata* ESTs in males (A) and females (B) at 3 different ages (0, 5 and 15 days) obtained by the *SOTArray* method is represented by these binary trees, in which the most similar patterns are clustered in a hierarchy of nested subsets. Grey scale designates relative transcript abundance in a given chronological age, with grey and white being the up and down-regulated, respectively.

tive expression level rates of each gene/EST are referenced in grey scale in Fig. 2. Within these differential expressed genes, one of the internal controls, the RpS21 gene, presented a differential expression pattern in females whereas the RpO showed a constant pattern.

Hierarchical clustering analysis of the expression pattern profiles showed differences between the males and females (Fig. 2a and 2b). With the SOTA of z-score vectors 5 main clusters with different expression patterns were constructed. Males and females showed different expression pattern for the same gene (EST). In males, for the three first clusters the transcripts were down- regulated. In the cluster 1 (7 transcripts), the lowest expression peak was reached by old flies. In this cluster, a vitellogenin-2 precursor (CC3P1C10) and a myosin heavy chain 3 (CC9P29D11) were included. In cluster 2 (13 cDNAs), which included a hexamerin (CC1P5G3) and a chorion protein s38 (CC3P9D4), the

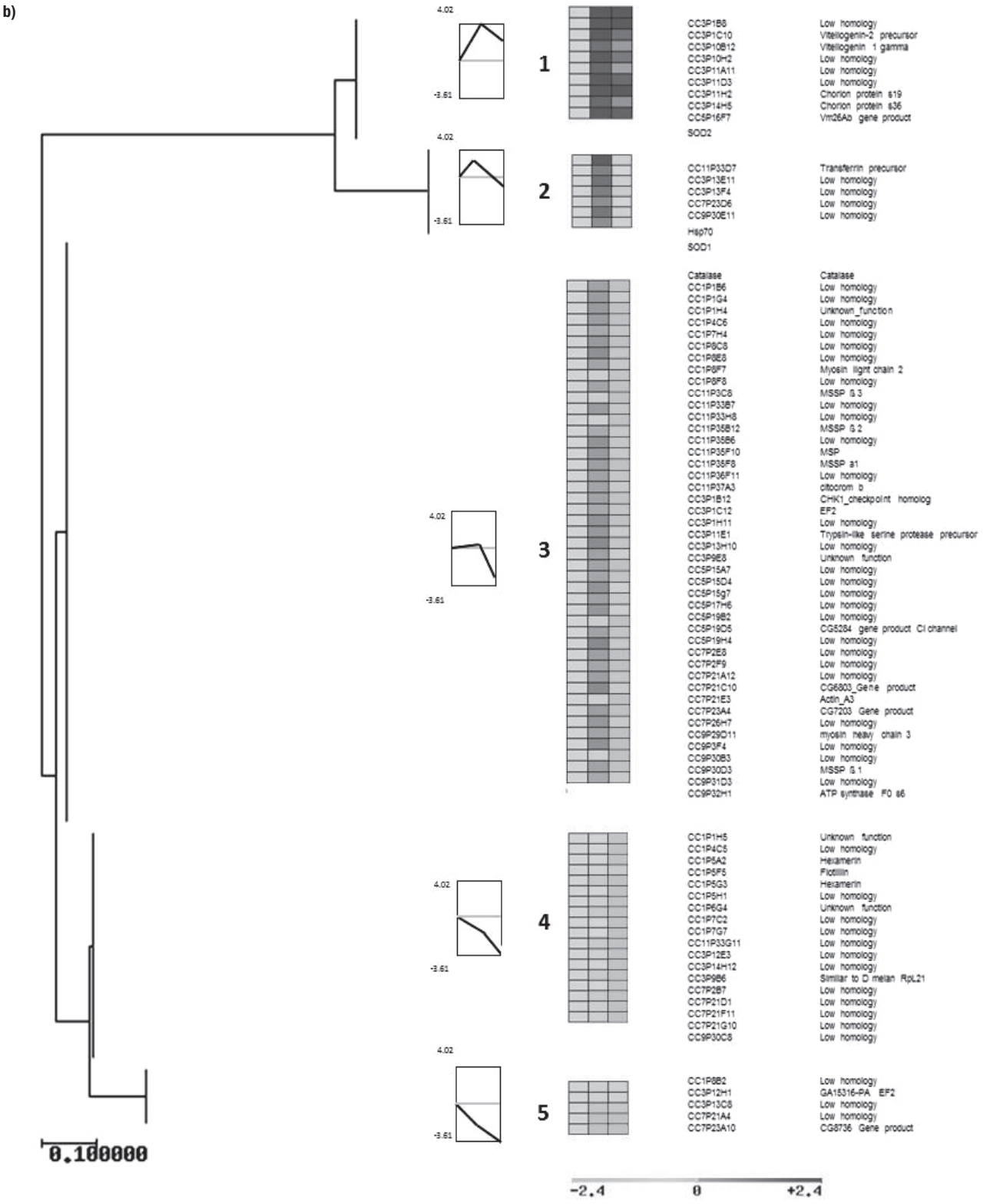

Figure 2 (cont.). Hierarchical clustering of differentially expressed *C. capitata* ESTs in males (A) and females (B) at 3 different ages (0, 5 and 15 days) obtained by the *SOTArray* method is represented by these binary trees, in which the most similar patterns are clustered in a hierarchy of nested subsets. Grey scale designates relative transcript abundance in a given chronological age, with grey and white being the up and down-regulated, respectively.

lowest expression was obtained for mature flies and then increased but slightly. The most abundant cluster (cluster 3), composed with 31 transcripts, included a cuticle protein (CC1P4A10), a hexamerin (CC1P5A2), a vitellogenin 1γ (CC3P10B12), a trypsin-like serine protease precursor (CC3P11E1), two chorion proteins (CC3P11H2 and CC3P14H5), the *actin* A3 (CC7P21E3) and the male specific serum protein (*mssp*) β1 (CC9P30D3) genes. The genes RpS21 and the *SOD2* were part of this cluster too. The expression of these transcripts remained constant between the immature and old flies decreasing from the interval between 0 and 5 days and then increasing. Cluster 4 (14 cDNAs) was composed by a transferrin precursor (CC11P33D7), a MSP (CC11P35F10), a cytochrome b (CC11P37A3), the CG5284 gene product Cl channel (CC5P19D5), and a ATP synthase F0 s8 (CC7P23C9). The expression of these transcripts decreased sharply at 5 day point but then increased reaching at 15 days old time point the same level expression than at 0 days. In this cluster, SOD1 was also found. The last cluster (11 transcripts) was the only up-regulated. For the two first time point, the expression was almost the same, but peaked considerably at 15 days old time point. Three *mssp* (β3, β2, α1) (CC11P3C8, CC11P35B12, CC11P35F8), the ATP synthase F0 s6 and the *hsp*70 were part of this group.

In females, 10 ESTs (cluster 1) including 2 vitellogenins (CC3P1C10-vitellogenin precursor and CC3P10B12-vitellogenin 1 γ), 2 chorion proteins (CC3P11H2-chorion protein s19 and CC3P3H5- chorion protein s36), and the gene SOD2 peaked at the 5 day time point followed by a slight decrease. The expression level of 7 other transcripts (cluster 2), including a transferring precursor (CC11P33D7) and the genes SOD1 and hsp70, had a similar expression pattern but less marked between time points. In the cluster 3 (the biggest one), transcripts presented a constant pattern expression for the two first time point, and then decreasing. This group, formed by 45 transcripts, included the 5 *mssp* gene products (CC11P3C8- *mssp* β3, CC11P35B12-*mssp* β2, CC11P35F8-*mssp* α1, CC11P35F10-*mssp*, CC9P30D3- *mssp* β1) and the elongation factor EF2 (CC3P1C12). In the cluster 4 (18 transcripts), the transcripts had their lowest peak at 15 days old time point. Two hexamerins (CC1P5A2 and CC1P5G3) and a flotillin were included. Finally, in the last cluster, the 5 ESTs displayed a similar decreasing expression pattern. An elongation factor product (CC3P12H1) belonged to this cluster.

Discussion

The set of genes obtained from the libraries suggests that a large number of genes are involved in ageing and/or maturity response pathways, although only a few of them have a known function. As result of libraries construction, the 57% of the unigenes obtained had protein homology to genes from the database; some of the identified ESTs are related to reproduction in females (*i.e.* vitellogenin, chorion protein), or in males (*i.e.* male specific serum protein) and their corresponding observed expression pattern are related to fertility status. Thus, the results obtained support the validity of the substracted libraries used, and these results were confirmed by macroarray expression analysis. Some of the genes that have been reported as ageing-related genes, such as SOD1, SOD2, *hsp*70 or catalase (Aigaki *et al.,* 2002) were not found in our libraries, but were sorted out by a differential expression pattern in the macroarray experiments. This could be due to the experimental design and medfly strain used, or due to the number of EST clones tested. In our experimental design for the EST libraries construction, the maturation process is more prone to be unveiled, as mating was allowed. Despite this limitation our results could be more realistic as some biological and cellular processes are also modulated by mating status, as demonstrated by Moshitzky *et al.* (2003) or by Gomulski *et al.* (2012). These authors have demonstrated that mating can modulate the biosynthesis of juvenile hormones in medfly females (the male specific serum proteins and peptides are the responsible of down-regulation of the juvenile hormones in the mated females when compared to virgin females of the same age). Regardless of common characteristics emerged between males and females libraries, some differences were also observed between them, suggesting the existence of sex-related expressed genes.

By using a combination of SSH and cDNA macroarray approach, we have successfully identified a set of genes with a modified expression pattern through ageing in term of reproduction in the medfly. The majority of the identified genes with known function in the databases were included in the cell development and response to chemical stimulus categories. Cell development class was mainly formed by vitellogenins and chorion proteins and response to chemical stimulus class, by male specific serum proteins.

In most animals, longevity is achieved at the expense of fertility (Carey, 2003). Vitellogenins (Vgs) are syn-

thesized by insects in the fat body and transported to the developing oocytes via the hemolymph (Wyatt & Davey, 1996). The primary function of this protein is to provide amino acids, carbohydrates, lipids for the developing embryo, but it has been also described as oxidative stress reducer, by scavenging free radicals and thereby prolonging life span (Seehuus *et al.,* 2006). Moreover, in some species Vgs are also produced by female immature stages, or even by males (Satyanarayana *et al.,* 1994; Yano *et al.,* 1994; Bellés, 2004; Raikhel *et al.,* 2004). The pattern of transcription obtained for the males and the females was the expected. In females, the production of Vgs increases during sexual maturation. Nevertheless, at 15 days-old a decrease was observed. In males, these genes are down-regulated, and the increased expression detected in 5 day-old adults could be due to their implication on other biological process (Bellés, 2004). Other group of transcripts included in the functional category cell development, present in female libraries, was those encoding for *chorion* genes. Changes in the expression of these genes in females are a general response to sexual maturity in insects. The egg represents a crucial stage of any insect life cycle and is linked to successful reproductive strategies. The organization, developmental regulation and evolution of clustered chorion gene family encoding chorion proteins have been extensively studied in Diptera. Six major *chorion* genes have been isolated from medfly located in two separate clusters (s36 and s38, s16, s19, s15 and s18 genes) (Konsolaki *et al.,* 1990; Tolias *et al.,* 1990; Vlachou *et al.,* 1997). These genes are under strict tissue regulation; both in space and time (see Vlachou *et al.,* 1997 and the references cited therein). The response to chemical stimulus class was only formed by male specific serum proteins (*mssp*). The *mssp* gene family in medfly consists of seven members classified in three subgroups according to the degree of the deduced polypeptide similarity: two *mssp* α, three *mssp* β, and two *mssp* γ. Five *mssps* have been characterized in the medfly (Katsoris *et al.,* 1990; Thymianou *et al.,* 1995). The synthesis of the *mssps* has been described to take place mainly during the first 5 days after adults' emergence (Thymianou *et al.,* 1998; Scolari *et al.,* 2012). Our data reveal that the main expression of these genes took place between the 5 and the 15 day after emergence, suggesting that male maturity in this medfly strain is reached later. We have also found that the ageing-maturing process in *C. capitata* females presents a marked expression pattern accompanied by the increase of transcription level of genes involved in reproduction followed by a reduction (vitellogenins and *chorion* genes), reaching the maximum peak expression at 5 days-old. In males, an increase in the expression level of some genes involved in reproduction (male specific serum proteins) is also observed, but it stars later in time (5 days-old adults) and the maximum has not been determined, because the maximum expression is obtained with 15 day old adults. The study of the expression pattern of the novel expressed genes obtained from the libraries as well as the expression pattern of the ageing-related genes included in this work, different from those described in other organisms and/or experimental conditions, may offer important clues in understanding the complexity of this process. Moreover, although we have been unable to assign a specific function to about 40 genes reported here, they would be of great interest when the medfly genome would be released by assigning a potential function to some of them.

Altogether, the use of the known genes, vitellogenins, chorion and male specific serum proteins, and these new candidates, would allow us to select in a future a transcriptional profiling protocol to determine the reproductive status and putative age of medflies early in spring (to get the first medfly generation of the year), as advisable for the SIT programmes. In this context, the data obtained could help us also in determination of optimal release age for sterile medfly males, but further research is needed.

The experimental design of this work, a medfly cohort in free-mating conditions to simulate the first medfly generation in wild population, has allowed us to sort out some age-maturation related genes as described, and these results have been confirmed by expression analysis by using macroarrays. Ageing-maturation is characterized by highly dynamic changes in the expression of many genes, which provides a powerful molecular description of the normal sources of variation in expression from experimental ageing process. The data reported in this study offer novel insights into the genes implied in maturity in medfly, some of them sex specific. Nevertheless, further studies on the biology and genes implicated in these processes are needed to designate candidate genes for reproduction-age biomarker test by a transcriptional profiling protocol. This work can be considered as the threshold for a more accurate differential expression pattern study, when the data of the ongoing *C. capitata* whole- genome sequencing will be available. The data provided will pave the way for new researches by given more information

of the "low homology" genes. All this information will allow developing a deeper expression evaluation by qRT-PCR at different ages and with wild flies.

Acknowledgments

We are thankful to Dr. A. Latorre (ICBIBE-UV) and Dr. P. Hernández (CIB-CSIC) for critical reading of early version of this manuscript. We are indebted to P. Vanaclocha and H. Montón for technical assistance. This work was supported by a grant of the European Commission (FOOD-CT-2003-506495, IAEA-FAO Research contract 15848) and by MICINN project AGL2010-21349-C02-02. V. San Andrés was recipient of an INIA PhD grant. B. Sabater-Muñoz has a post-doctoral contract of the INIA-CCAA programme.

References

Aigaki T, Seong K, Matsuo T, 2002. Longevity determination genes in *Drosophila melanogaster*. Mech Ageing Develop 123: 1531-1541.

Alberola TM, García-Martínez J, Antúnez O, Viladevall L, Barcelo A, Arino J, Pérez-Ortin JE, 2004. A new set of DNA macrochips for the yeast *Saccharomyces cerevisiae*: features and uses. Inter Microbiol 7: 199-206.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipmann DJ, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 25(17): 3389-3402.

Bellés X, 2004. Vitellogenesis directed by juvenile hormone. In: Reproductive biology of invertebrates, Vol. XII, Part B: Progress in vitellogenesis (Raikhel AS, ed). John Wiley & Sons Inc, NY. pp: 157-198.

Camin V, Baker P, Carey J, Valenzuela J, Arredondopeter R, 1991. Biochemical age-determination for adult Mediterranean fruit-flies (Diptera, Tephritidae). J Econ Entomol 84: 1283-1288.

Carey JR, 2003. Longevity: the biology and demography of life span. Princeton Univ Press, Princeton, NJ, USA. 278 pp.

Carey JR, Papadopoulos NT, Kouloussis NA, Katsoyannos BI, Müller HG, Wang JL, Tseng YK, 2006. Age-specific and lifetime behavior patterns in *Drosophila melanogaster* and the Mediterranean fruit fly, *Ceratitis capitata.* Exp Geront 41(1): 93-97.

Carey JR, Papadopoulos NT, Müller H-G, Katsoyannos BI, Kouloussis NA, Wang JL, Wachter K, Yu W, Liedo P, 2008. Age structure changes and extraordinary lifespan in wild medfly populations. Aging Cell 7(3): 426-437.

Cook PE, Hugo LE, Iturbe-Ormaetxe I, Williams CR, Chenoweth SF, Ritchie SA, Ryan PA, Kay BH, Blows MW, O'Neill SL, 2006. The use of transcriptional profiles to predict adult mosquito age under field conditions. P Natl Acad Sci USA 103: 18060-18065.

Cook PE, Hugo LE, Iturbe-Ormaetxe I, Williams CR, Chenoweth SF, Ritchie SA, Ryan PA, Kay BH, Blows MW, O'neill SL, 2007. Predicting the age of mosquitoes using transcriptional profiles. Nature Prot 2: 2796-2806.

Desena ML, Clark JM, Edman JD, Symington SB, Scott TW, Clark GG, Peters TM, 1999. Potential for aging female *Aedes aegypti* (Diptera: Culicidae) by gas chromatographic analysis of cuticular hydrocarbons, including a field evaluation. J Med Entomol 36: 811-823.

Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD, 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. P Natl Acad Sci USA 93: 6025-6030.

Gerade BB, Lee SH, Scott TW, Edman JD, Harrington LC, Kitthawee S, Jones JW, Clark JM, 2004. Field validation of *Aedes aegypti* (Diptera: Culicidae) age estimation by analysis of cuticular hydrocarbons. J Med Entomol 41: 231-238.

Gomulski LM, Dimopulos G, Xi Z, Scolari F, Gabrieli P, Siciliano P, Clarke AR, Malacrida AR, Gaspieri G, 2012. Transcriptome profiling of sexual maturation and mating in the Mediterranean fruit flies, *Ceratitis capitata*. PLOS One 7(1): e30857.

Hendrichs J, Vreysen MJB, Enkerlin WR, Cayol JP, 2005. Strategic options in using sterile insects for area-wide integrated pest management. In: Sterile insect technique. Principles and practice in area-wide integrated pest management (Dyck VA, Hendrichs J, Robinson AS, eds). Springer, Dordrecht, The Netherlands. pp: 563-600.

Herrero J, Al-Shahrour F, Díaz-Uriarte R, Mateos A, Vaquerizas JM, Santoyo J, Dopazo J, 2003. GEPAS: a web-based resource for microarray gene expression data analysis. Nucl Acids Res 31: 3461-3467.

Katsoris PG, Mavroidis M, Mintzas AC, 1990. Identification and characterization of male specific serum proteins in the Mediterranean fruit fly *Ceratitis capitata*. Insect Biochem 20(7): 653-657.

Kim SK, 2008. Genome-wide views of aging gene networks. In: Molecular biology of aging (Guarente LP, Partridge L, Wallace DC (eds). Cold Spring Harbor Lab Press, Cold Spring Harbor, USA. 610 pp.

Kirkwood TBL, Austad SN, 2000. Why do we age? Nature 408: 233-238.

Konsolaki M, Komitopoulou K, Tolias PP, King DL, Swimmer C, Kafatos FC, 1990. The chorion genes of the medfly, *Ceratitis capitata*, I: Structural and regulatory conservation of the s36 gene relative to two *Drosophila* species. Nucl Acid Res 18(7): 1731-1737.

Liquido NJ, Shinoda LA, Cunningham RT, 1991. Host plants of the Mediterranean fruit fly (Diptera: Tephritidae): an annotated world review. Misc Pub Entomol Soc Am 77: V-52.

Magaña C, Hernández-Crespo P, Brun-Barale A, Couso-Ferrer F, Bride M, Castañera P, Feyereisen R, Ortego F,

2008. Mechanisms of resistance to malathion in the medfly *Ceratitis capitata.* Insect Biochem Mol Biol 38: 756-762.

Malacrida AR, Gomulski LM, Bonizzoni M, Bertin S, Gasperi G, Guglielmino CR, 2007. Globalization and fruitfly invasion and expansion: the medfly paradigm. Genetica 131(1): 1-9.

McCarroll SA, Murphy CT, Zou S, Pletcher SD, Chin CS, Jan YN, Kenyon C, Bargmann CI, Li H, 2004. Comparing genomic expression patterns across species identifies shared transcriptional profile in aging. Nature Genet 36: 197-204.

Moshitzky P, Miloslavski I, Aizenshtat Z, Applebaum SW, 2003. Methyl palmitate: a novel product of the medfly (*Ceratitis capitata*) corpus allatum. Insect Biochem Mol Biol 33(12): 1299-1306.

Mullens BA, Lehane MJ, 1995. Fluorescence as a tool for age determination in *Culicoides variipennis sonorensis* (Diptera: Ceratopogonidae). J Med Entomol 32: 569-571.

Novoseltsev VN, Arking R, Carey JR, Novoseltseva JA, Yashin AI, 2005. Individual fecundity and senescence in *Drosophila* and medfly. J Geront Series A: Biol Sci Med Sci 60(8): 953-962.

Papadopoulos NT, Carey JR, Katsoyannos BI, Kouloussis NA, Müller HG, Liu X, 2002. Supine behaviour predicts time-to-death in male Mediterranean fruit flies. Proc Royal Soc London: Biol Sci 269: 1633-1637.

Penilla RP, Rodríguez MH, López AD, Viader-Salvado JM, Sánchez CN, 2002. Pteridine concentrations differ between insectary-reared and field-collected *Anopheles albimanus* mosquitoes of the same physiological age. Med Veter Entomol 16: 225-234.

Raikhel AS, Brown MR, Bellés X, 2004. Hormonal control of reproductive processes. In: Comprehensive molecular insect science, Vol. 3 (Gilbert LI, Iatrou K, Gill SS, eds). Elsevier, Oxford, UK. pp: 433-491.

Sabater-Muñoz B, Legeai F, Rispe C, Bonhomme J, Dearden P, Dossat C, Duclert A, Gauthier JP, Ducray DG, Hunter W *et al*., 2006. Large-scale gene discovery in the pea aphid *Acyrthosiphon pisum* (Hemiptera). Genome Biol 7(3): R21.

Sambrook J, Fritsch EF, Maniatis T, 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, USA. 2344 pp.

San Andrés V, Urbaneja A, Sabater-Muñoz B, Castañera P, 2007. A novel molecular approach to assess mating success of sterile Medfly males in SIT programs. J Econ Entomol 100(4): 1444-1447.

Satyanarayana K, Bradfield JY, Bhaskaran G, Dahm KH, 1994. Stimulation of vitellogenin production by methoprene in prepupae and pupae of *Manduca sexta*. Arch Insect Biochem Physiol 25: 21-37.

Scolari F, Gomulski LM, Ribeiro JM, Siciliano P, Meraldi A, Falchetto M, Bonomi A, Manni M, Gabrieli P, Malovini A, Bellazzi R, Aksoy S, Gasperi G, Malacrida AR, 2012. Transcriptional profiles of mating-responsive genes from testes and male accessory glands of the Mediterranean fruit fly, *Ceratitis capitata.* PLOS One 7(10): e46812.

Seehuus SC, Norberg K, Gimsa U, Krekling T, Amdam GV, 2006. Reproductive protein protects sterile honey bee workers from oxidative stress. P Natl Acad Sci USA 103: 962-967.

Staden R, Beal KF, Bonfield JK, 2000. The Staden package, 1998 In: Methods in molecular biology, vol. 132, Bioinformatics: methods and protocols (Misener S, Krawetz S, eds.. Humana Press Inc, Totowa, NJ, USA. pp: 115-130.

Thymianou SP, Chrysanthis G, Petropoulou KA, Mintzas AC, 1995. Developmentally regulated biosynthesis of two male-specific serum protein in the fat body of the Mediterranean fruit fly, *Ceratitis capitata.* Insect Biochem Mol Biol 25(8): 915-920.

Thymianou SP, Mavroidis M, Konsolaki M, Komitopoulou K, Zacharopoulou A, Mintzas AC, 1998. Cloning and characterization of a cDNA encoding a male-specific serum protein of the Mediterranean fruit fly, *Ceratitis capitata*, with sequence similarity to odorant-binding proteins. Insect Mol Biol 7(4): 345-353.

Tolias PP, Konsolaki M, Komitopoulou K, Kafatos FC, 1990. The *chorion* genes of the medfly, *Ceratitis capitata.* II. Characterization of three novel cDNA clones obtained by differential screening of an ovarian library. Develop Biol 140: 105-112.

Tyndale-Biscoe M, 1984. Age-grading methods in adult insects a review. Bul Entomol Res 74: 341-377.

Vlachou D, Konsolaki M, Tolias PP, Kafatos FC, Komitopoulou K, 1997. The autosomal *chorion* locus of the medfly *Ceratitis capitata*. I. Conserved synteny, amplification and tissue specificity but sequence divergence and altered temporal regulation. Genetics 147: 1829-1842.

White IM, Elson-Harris MM, 1992. Fruit flies of economic significance: their identification and bionomics. CAB Int Publ, The Australian Center for International Agricultural Research, Eastbourne, Australia.

Wyatt GR, Davey KG, 1996. Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects. Adv Insect Physiol 26: 1-155.

Yano K, Sakurai MT, Watabe S, Izumi S, Tomino S, 1994. Structure and expression of mRNA for vitellogenin in *Bombyx mori*. Biochim Biophys Acta 1218 (1): 1-10.